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**Pigment patterns in adult fish result from superimposition of two largely independent pigmentation mechanisms**

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Short title: Dual pigment patterning mechanisms in zebrafish

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## ABSTRACT

Dorso-ventral pigment pattern differences are the most widespread pigmentary adaptations in vertebrates. In mammals, this pattern is controlled by regulating melanin chemistry in melanocytes using a protein, ASIP. In fish, studies of pigment patterning have focused on stripe formation, identifying a core striping mechanism dependent upon interactions between different pigment cell types. In contrast, mechanisms driving the dorso-ventral countershading pattern have been overlooked. Here, we demonstrate that, in fact, zebrafish utilize two distinct adult pigment patterning mechanisms - an ancient dorso-ventral patterning mechanism, and a more recent striping mechanism based on cell-cell interactions; remarkably, the dorso-ventral patterning mechanism also utilizes ASIP. These two mechanisms function largely independently, with resultant patterns superimposed to give the full pattern.

**Keywords** Agouti, pigment pattern formation, *Asip1*, *Dct*, *Tyrp1b*, *Xdh*, *Ltk*, *Mitfa*, melanophore, melanocyte, iridophore, chromatophore, transgenic, zebrafish.

56     **SIGNIFICANCE**

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58     Pigment pattern formation is a classic problem in biology amenable to a genetic approach in  
59     mammals and fish. In fish, most studies examined stripe formation, whereas our study reveals  
60     graded Agouti-signalling peptide (ASIP) as underlying the previously ignored counter-  
61     shading. This demonstrates that ASIP-dependent counter-shading is conserved between  
62     mammals and fish, an unexpected molecular conservation given the apparently distinct  
63     cellular bases of pigment pattern formation in these groups.

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## INTRODUCTION

Pigment patterns allow animals to distinguish among individuals, groups and species and are an essential characteristic for the survival of most animals in wild populations. These patterns allow appropriate adaptation to the environment, often having crucial roles as components of courtship behaviours, or making hunting easier, or providing camouflage against potential predators. A prime example of an evolutionarily conserved pigment pattern is dorso-ventral countershading - a dark dorsal region contrasting with a pale ventrum - a pattern that is easily recognizable in most vertebrate taxa. These patterns are dependent upon pigment cells, or chromatophores, that synthesize or accumulate pigments or crystalline compounds (Kelsh, 2004). All vertebrate integumentary chromatophores arise from neural crest cells (NCCs), multipotent embryonic cells that also give rise to other cell types, such as neurons and glia of the peripheral nervous system and elements of the craniofacial skeleton (Le Douarin and Kalcheim, 1999; Maury and Jacobson, 1989). Mammalian species have only one type of chromatophore, the melanocyte, which synthesizes eumelanin (black/brown pigment) or pheomelanin (yellow/red pigment) under the control of two main loci, *extension* and *agouti*. The *extension* locus encodes the receptor for  $\alpha$ -Melanocyte Stimulating Hormone ( $\alpha$ -MSH), Melanocortin 1 Receptor (*MC1R*), which promotes eumelanin synthesis when stimulated (Robbins et al., 1993). The agouti protein, termed agouti-signalling protein (ASIP) in species other than mouse, is encoded by the *Agouti* locus and antagonizes MSH effects at MC1R, resulting in inhibition of eumelanin synthesis coupled to a switch to pheomelanin synthesis (Bultman et al., 1992; Michaud et al., 1993; Miller et al., 1993). In mammals, dorso-ventral countershading is driven by regulation of agouti expression and consequent modification of the melanin type in differentiated melanocytes. The differential usage of alternative promoters regulates the spatial and temporal expression of the *ASIP* gene that, in turn, controls positional and temporal synthesis of eumelanin *versus* pheomelanin. The sustained expression of ASIP protein in the ventral region is responsible for the dorso-

95 ventral pigment pattern in which the ventrum is light (pheomelanin) while the dorsum is more  
 96 darkly colored (eumelanin) (Millar et al, 1993 and Vrieling et al, 1994). Similarly, a temporal  
 97 pulse of *agouti* expression during a window of the hair growth cycle results in the classical  
 98 black-yellow-black banded ('agouti') pattern in the dorsal fur of many mammalian species  
 99 (Vrieling et al., 1994). Studies in rodents indicate that the local production of agouti protein  
 100 also appears to contribute to changes in pigment pattern evolution through developmental  
 101 modifications (Manceau et al., 2011).

102 In poikilotherms, including fish, dorso-ventral countershading is achieved by means of a  
 103 mechanism distinct from the differential differentiation of melanocytes in mammals. In fish,  
 104 the mechanism involves a patterned distribution of different types of pigment cells or  
 105 chromatophores, with the light-absorbing (melanocytes, often known as melanophores) and  
 106 light-reflecting (iridophores) chromatophores mostly distributed in the dorsal and ventral  
 107 areas, respectively (Bagnara and Matsumoto, 2006). Experimental data in amphibian and fish  
 108 species support the hypothesis that the dorso-ventral pigment pattern results from interaction  
 109 between two factors, melanization inhibition factor (MIF) and  $\alpha$ -MSH. Although so far MIF  
 110 has never been purified, it has been suggested that MIF is a factor present in tissue extracts  
 111 from ventral skin that reduces melanocyte number and increases the number of iridophores in  
 112 the ventrum (Fukuzawa and Ide, 1988; Bagnara and Fukuzawa, 1990; Zuasti, 2002).  
 113 Amphibian MIF blocks the stimulation of melanization provoked by  $\alpha$ -MSH in neural  
 114 explants of *Xenopus* (Fukuzawa and Bagnara, 1989). Similarly, amphibian MIF has been  
 115 shown to block  $\alpha$ -MSH-induced tyrosine hydroxylase and dopa oxidase activity, both key  
 116 enzymes in the melanogenic pathway, in malignant mouse melanocytes (López-Contreras et  
 117 al., 1996). Our previous studies have lead us to propose that *Asip1* corresponds to the  
 118 uncharacterized non-mammalian MIF (Cerdá-Reverter et al., 2005; Guillot et al., 2012;  
 119 Darias et al., 2013). Thus, *Asip1* is predominantly expressed in the ventral skin with residual

120 levels in the dorsal skin (Cerdá-Reverter et al., 2005; Guillot et al., 2012). Secondly, the  
121 injection of capped *Asip1* mRNA induces lightening of dorsal skin in flatfish whereas white  
122 skin patches in the dorsal area of pseudoalbino flatfish overexpress *Asip1* mRNA. Thirdly,  
123 *Asip1* mRNA injection results in lowered expression of transcripts for tyrosinase-related  
124 protein-1 (*Tyrp1*), a key enzyme of melanin synthesis, and *Tyrp1* gene expression is  
125 downregulated also in dorsal white patches (Guillot et al., 2012). Finally, goldfish *Asip1* is  
126 able to block the  $\alpha$ -MSH-induced melanosome movement in the medaka scales via MC1R  
127 (Cerdá-Reverter et al., 2005). If our proposal that MIF is in fact *Asip1* is true, this suggests  
128 the exciting hypothesis that ASIP has a conserved function, but that it may act through a  
129 different cellular mechanism in mammals and in fish.

130 Zebrafish is widely used as a model organism for developmental studies and there are now a  
131 number of studies reporting gene regulatory networks underlying the development of pigment  
132 cells, both in the embryo (Greenhill et al., 2011) and in the adult (Kelsh et al., 2009; Parichy  
133 et al., 2009). Adult pigment patterns derive from adult pigment stem cells, including  
134 melanocyte stem cells (MSCs), set aside from the neural crest in the embryo but  
135 differentiating during metamorphosis (Budi et al., 2011; Dooley et al., 2012). In adult  
136 zebrafish, most of these studies have focused on determining the mechanism underlying the  
137 intrinsically interesting, but evolutionarily labile, formation of stripes (Quigley and Parichy,  
138 2002). These studies have identified a core striping mechanism dependent upon interactions  
139 between different pigment cell-types (Patterson and Parichy, 2013; Frohnofer et al., 2013;  
140 Krauss et al., 2013; Maderspacher and Nüsslein-Volhard, 2003; Yamaguchi et al., 2007; Inaba  
141 et al., 2012). These studies overlook the fact that zebrafish also display a dorso-ventral  
142 countershading pattern, conserved across the vertebrates. We hypothesized that zebrafish  
143 utilizes two distinct adult pigment patterning mechanisms – an ancient dorso-ventral  
144 patterning mechanism, and a more recent striping mechanism based on cell-cell interactions;  
145 these mechanisms function largely independently and the resultant patterns are then



superimposed to give the full pattern. We hypothesized that the dorso-ventral pattern might result from a dorso-ventrally graded expression of *Asip1* that regulates the balance of the different types of pigment cells derived from adult pigment stem cells. In order to test this hypothesis, we first show that *Asip1* expression in wild-type (WT) zebrafish skin shows a dorso-ventral gradient, high ventrally; we show too that this gradient is established at a time coincident with the onset of metamorphosis, and that expression in adults is predominantly restricted to the iridophores. Then we assess the effects of disruption of this gradient, by generating transgenic lines overexpressing *Asip1* under the control of a constitutive promoter. These fish exhibit lightening of the dorsal skin; melanocyte counts and quantitation of molecular markers show that this results from inhibition of melanocyte differentiation, but also from a partial reduction of melanocyte numbers. Concomitantly, extensive iridophores become visible in the dorsal area of *Asip1* transgenic zebrafish that together with the lack of dorsal melanophores gives a ventralised appearance to the dorsal region. In contrast, we see only relatively modest effects on the core striped pattern, with most stripes still visible. We conclude that adult zebrafish utilize two superimposed pigment patterning mechanisms to generate their characteristic pattern.

## RESULTS

### Temporal and spatial characterisation of *Asip1* expression

To investigate the role of *Asip1* in zebrafish pigment pattern formation, we first examined the temporal and tissue distribution of *Asip1* expression by RT-PCR (see Fig. S1). *Asip1* was detected at all stages examined (Fig. S1A). In adult fish, *Asip1* mRNA was widespread: we found expression primarily in eye, dorsal and ventral skin, heart, fat and muscle and was present at lower levels in intestine, liver, brain, gill and kidney; and at extremely low levels in the gonads (Fig. S1B). Because RT-PCR has inherent limitations, particularly those that result in biases in the template-to-product ratios of target sequences, we used quantitative real time

PCR (qRT-PCR) to characterize further the transcriptional regulation of *Asip1* during zebrafish development (Fig.1A). qRT-PCR showed that a high level of *Asip1* mRNA was associated with larval to adult metamorphosis (2–4 weeks post fertilisation; Johnson et al., 1995) (Fig. 1A), the time when the larval pigment pattern established during the first few days of development is gradually replaced by the adult pigment pattern.

We then used whole-mount mRNA *in situ* hybridization to examine where *Asip1* mRNA was expressed at different stages. In the embryo, we saw no detectable expression at 30 hours post-fertilisation (hpf), expression was first detectable by this method in 3 day post-fertilisation (dpf) embryos, when the developing opercle was the initial site of expression (Fig. 2 A,B,C). At 6 dpf, *Asip1* transcripts were still present in the opercle, but not seen elsewhere. In contrast, in adult (210 dpf) zebrafish, *Asip1* messenger RNA was detectable in the dermal iridophores located in the most ventral interstripe (interstripe X1V) (Fig. 2G) and stripe (stripe 2V) (Fig.2H) but not in the dermal iridophores located in the most dorsal stripes and interstripes (data not shown). Additionally, *Asip1* transcripts were also expressed in the intraperitoneal membrane region where a reflecting iridophore layer is located (Fig. 2F), but otherwise were not seen in tissues other than the skin.

#### **Dorso-ventral gradient of agouti expression**

Our detection of *Asip1* expression in the skin of adult fish (Fig. 2 and Fig. S1B), was consistent with *Asip1* having a possible role in adult pigment pattern formation, and the whole mount *in situ* hybridization studies indicated that expression might vary spatially. To test this quantitatively, we investigated whether and when there were dorso-ventral differences in expression levels by qRT-PCR. We measured *Asip1* transcript levels in skin from dorsal and belly regions of 15, 30 and 60 dpf zebrafish (Fig. 1B). Dorso-ventral differences in *Asip1* expression levels were found at 30 and 60 dpf, but were not significantly different at 15 dpf. The highest *Asip1* transcript levels were found in the belly (Fig.1B)

In order to precisely characterize the dorso-ventral differences in *Asip1* expression levels, we determined *Asip1* transcript levels in adult skin from different dorso-ventral positions, specifically, dorsal, all body stripes (2D, 1D, 1V and 2V) and belly skin regions (Fig. 3A,I). Figure 3K shows the expression levels of *Asip1* in skin samples obtained along the dorso-ventral axis in wild-type adult zebrafish. The highest levels of expression of *Asip1* were found in the belly ( $p < 0.001$ ); however, *Asip1* transcripts showed a gradual decrease from the belly to the dorsal midline of the fish. This striking observation leads us to hypothesize that *Asip1* activity might contribute to dorso-ventral differences in zebrafish pigment pattern formation. Conversely, although *Asip1* mRNA expression was also detected in WT zebrafish fins. No dorso-ventral gradient expression was detected in all these tissues (see Fig. S2).

#### **Overexpression of *Asip1* modifies the zebrafish pigment pattern**

To investigate whether dorso-ventral *Asip1* expression levels regulate pigment pattern, we studied whether changes in the concentration and distribution of *Asip1* expression were accompanied by phenotypic changes in the pigment pattern. Specifically, we predicted that overexpression of *Asip1* in dorsal regions of the skin would result in a ventralisation (i.e. a lightening due to an increased ratio of iridophores to melanocytes) of the pigment pattern. We generated three independent transgenic zebrafish lines (*Tg(Xla.Eef1a1:Cau.Asip1)iim03*, *Tg(Xla.Eef1a1:Cau.Asip1)iim04*, *Tg(Xla.Eef1a1:Cau.Asip1)iim05*) that overexpress goldfish *Asip1* using the constitutive EF1-alpha promoter (see Fig. S2). All of these fish showed a distinct pigment pattern phenotype as adults (Fig. 3B). In the following analyses, we focus on line *Tg(Xla.Eef1a1:Cau.Asip1)iim05*. To test whether this correlated with an expected homogenization of the *Asip1* gradient due to the transgene, *Asip1* transgene expression levels were determined in six skin samples from the most dorsal to the most ventral regions in adult fish (Fig. 3J) by qRT-PCR (Fig 3L). The domain of transgene expression extended throughout the dorso-ventral axis, and no significant differences were found in the levels of expression

between these positions. We conclude that the transgene generates an essentially homogeneous distribution of *Asip1* gene expression in the skin, eliminating the striking gradient seen in WT siblings. Furthermore, comparing the normalized expression levels in each genotype, we note that the levels of *Asip1* expression throughout the skin of transgenic fish are equivalent to those in the ventral regions of a WT fish (Fig. 3K, L).

Compared with WT siblings (Fig. 3C, G), the transgenics had reduced pigmentation over the entire dorsal region (Fig. 3D), including the head (Fig. 3H). This dramatic hypomelanisation results from an almost complete absence of melanin from the dorsal scale melanophores, and was also prominent in the body stripes, with 2D absent and the others showing reduced melanin (Fig. 3B, F). In place of stripe 2D, the transgenics show an extensive dorsal extension of the dorsalmost band of iridophores (interstripe) dorsal to the remaining stripe 1D (Fig. 3B, F). Nevertheless, other aspects of the pigment pattern are unchanged, with fin pigment pattern, opercula iridophores, pigmentation of the eye (both choroidal and retinal pigmented epithelium) occurring in a manner indistinguishable from WT fish. The pattern seen can reasonably be described as a ventralisation, consistent with the qRT-PCR results showing that transgenic fish attain a level of *Asip1* expression typical of WT ventral levels.

Embryonic, metamorphic and adult pigment patterns can be distinguished at different stages of the zebrafish life cycle, mainly by an increase in the number of melanophores and changes in their distribution (Kelsh, 2004; Parichy et al., 2009). In order to identify which of these patterns were affected in transgenic fish, melanophores were counted during larval (5 dpf, SL 3 mm), metamorphic (15 dpf, SL 6.3 mm) and two adult stages (60 dpf, SL 13 mm and 210 dpf, SL 25 mm)(Fig. 4 and Fig. S4). No differences was observed in the number of dorsal stripe melanophores in fish at 5 and 15 dpf, nor in lateral stripe melanophores in the horizontal myoseptum at 15 dpf (see Fig. S4). Whole-mount *in situ* hybridization was performed to determine whether *Asip1* overexpression affected the expression pattern of *microphthalmia-related transcription factor a* (*Mitfa*), *xanthine dehydrogenase* (*Xdh*), and

*leukocyte tyrosine kinase (Ltk)*, early markers for melanophores, xanthophores and iridophores respectively (Parichy et al., 2000; Lopes et al., 2008). Although *Asip1* overexpression is widespread in the transgenic line even as early as embryonic stages (Fig. S3), we saw no differences in the expression pattern of any of these markers at 24-30hpf, consistent with the normal embryonic pigment pattern of the transgenic fish (data not shown). In contrast, transgenic fish at 60 and 210 dpf showed pronounced pigment defects. Fig. 4 shows the melanophore distribution in representative WT (A) and transgenic (B) fish at 210 days of life. To enable accurate quantitation of melanophore numbers, fish were treated with epinephrine and fixed in 4% paraformaldehyde, which removes the pigments of xanthophores and iridophores but does not affect melanin. At 60 dpf, the number of skin melanophores in transgenic fish was decreased by 61% in the head, 66% in the dorsum and 35% in stripe 1D on the trunk compared to the control group ( $p<0.001$ ); we saw no differences in the melanophore number in the anterior and posterior regions of stripe 1V nor belly (Fig. 4C). At 210 days of life, a similar pattern of decreased number of melanophores in the dorsum and stripe 1D persisted, and the transgenic fish lacked the additional 2D melanophore stripe, having only a few scattered melanophores (Fig. 4B,D). Control individuals of the same SL and age as transgenic fish showed the normal 2D band (Fig. 4A, C). Thus, the increase in *Asip1* expression consistently reduced the number of melanophores, but this effect was restricted to dorsal regions of metamorphic transgenic fish.

#### **Pattern changes result from decreased melanocyte numbers, as well as decreased melanisation**

Next we asked whether the modified pigment patterns observed in transgenic fish were due to changes in melanoblast differentiation, especially inhibition of the melanin synthesis pathway, or due to absence of melanophores. *Mitfa* is a critical regulator of melanophore fate specification and encodes a transcription factor required for expression of melanin-synthesis

involved genes such as *Dct* and *Tyrp1b*, but also for ongoing maintenance of the melanocyte phenotype (Lister et al., 1999; Johnson et al., 2011).

We analysed the levels of expression of *Mitfa*, *Dct* and *Tyrp1b* in skin from the dorsum and from dorsal (2D, 1D) and ventral (1V, 1D) dark stripes and belly in control and *Asip1* transgenic fish by qRT-PCR (Fig. 5). Both *Tyrp1b* and *Dct* (*Tyrp2*) code for enzymes that participate in the melanogenic pathway, but *Dct* in particular is also an early marker of melanoblast differentiation (Lister et al., 1999; Kelsh and Eisen, 2000). Figure 5B shows the levels of expression of *Dct* along the dorso-ventral axis in the skin of control and transgenic fish. In transgenic fish, *Dct* expression was decreased by 93.4% in the dorsum and by 84.2% in 2D stripes in comparison with the same regions in WT fish. *Dct* expression in 1D, 1V and 2V stripes and belly did not differ significantly between transgenic and WT fish. The expression pattern of *Tyrp1b* (Fig. 5C) was similar to that of *Dct*. Thus, in the transgenic group, the expression of *Tyrp1b* was 2.63 times less in dorsum and 3.7 times less in the “virtual” 2D stripe than in the control group. In dark stripes 1D, 1V and 2V, *Tyrp1b* expression was similar in the two groups. Thus, consistent with the dorsal reduction in melanisation, we see a dramatic reduction of expression of melanophore differentiation genes in dorsal, but not ventral, regions of transgenic fish.

Figure 5A shows the expression levels of *Mitfa* in skin samples obtained along the dorso-ventral axis in zebrafish adults. In wild-type control fish, the highest levels of expression of *Mitfa* were found in the dorsum ( $p < 0.001$ ), but *Mitfa* transcripts showed a gradual decrease from the dorsum to the belly of the fish, thus showing an evident dorso-ventral gradient expression in adult WT zebrafish. In transgenic fish, *Mitfa* expression was decreased by 50% in the dorsum with no significant difference in the other skin regions in comparison with the same regions in WT fish. Therefore, comparing the normalized expression levels of *Mitfa* in each genotype, we conclude that the disruption of the *Asip1* dorso-ventral expression gradient found in the transgenic fish produces a disruption of the striking *Mitfa* gradient expression

seen also in WT siblings. Furthermore, we note the good correlation between *Mitfa* expression levels (Fig. 5A) and melanophore numbers (Fig. 4D), suggesting that most, if not all, melanophores in the transgenics are pigmented, and that their number is indeed decreased in dorsal regions of the transgenic lines. Comparing the data for *Dct* and *Tyrp1b*, we note that in stripe 2D, *Mitfa* is not significantly changed, whereas the melanocyte differentiation genes are dramatically reduced in good correlation to the counts of melanised melanophores; we interpret these data as indicating that in this region elevated *Asip1* levels result in partial inhibition of melanocyte differentiation. In contrast, in the dorsalmost region, where *Dct*, *Tyrp1b* and *Mitfa* are all at least very substantially reduced, we suggest that *Asip1* overexpression results in a decrease in the number, as well as the differentiation, of melanophores in transgenic fish.

#### **Early markers of xanthophores and iridophores are unaffected by *Asip1* overexpression**

The pigment pattern of zebrafish is composed not only of melanophores but also of xanthophores and iridophores. Unlike melanophores, it is particularly difficult to distinguish individual xanthophores and iridophores, so that it is difficult to count them in post-embryonic stages. Therefore, to determine whether xanthophores and iridophores in transgenic fish were affected, we analysed the levels of expression of *Xdh*, a gene encoding an enzyme required for the synthesis of yellow–orange pteridine pigments (Parichy et al., 2000), and *Ltk*, an early and persistent marker of the iridophore lineage (Lopes et al., 2008) by qRT-PCR in the skin of control and transgenic adult fish. Both *Xdh* and *Ltk* were detected in dorsum and belly and also in dark stripes in control and transgenic fish (Fig. 5D, E), with no significant difference in their expression levels at this stage. We conclude that numbers of each of these cell-types are likely to be largely unchanged in transgenic fish.

#### **DISCUSSION**

In this study, we show a dorso-ventral gradient of *Asip1* expression in the skin of adult zebrafish, with lower levels in the dorsum, remaining constant until the horizontal myoseptum, and increasing gradually to reach a maximum in the belly. This expression pattern correlates with the dorso-ventral pigment pattern showing darkly colored skin in the dorsal area and light ventrum. Disruption of the dorso-ventral expression gradient in transgenic zebrafish overexpressing *Asip1* results in a modification of the dorso-ventral pigment pattern. Transgenic fish exhibit a drastic reduction in the number of differentiated melanocytes in the dorsal region, due to the absence of most scale-associated melanophores as well as most of the melanophores in the dorsal stripes (1D and 2D) and the dorsal head of adult zebrafish. The lack of melanised cells in the dorsal region enhances the visibility of the underlying iridophores, thus giving a ventral-like aspect to the dorsal skin of transgenic zebrafish. Careful quantitation of transcripts for two key melanogenic enzymes, *Dct* and *Tyrp1b*, and of the transcription factor *Mitfa* which is crucial for specification and maintenance of melanocytes, clearly indicates that this reduction in the number of differentiated melanophores results both from a reduction in the number of dorsal melanocytes and a reduction in the differentiation (melanisation) of the remaining cells. Our data thus shows that *Asip1* function is crucial for development of the dorso-ventral counter-shaded pattern in adult zebrafish.

Our data suggest that zebrafish adult pigment pattern formation involves two processes. To date, previous work has focused on a stripe-forming mechanism, that is dependent upon interactions between xanthophores and melanophores (Kelsh et al., 2009; Parichy 2009), but with recent work beginning to uncover a key role for iridophores in this mechanism too (Patterson and Parichy, 2013; Frohnhöfer et al., 2013; Singh et al, 2014). Here, we have identified a role for *Asip1* in generating an underlying dorso-ventral (countershading) mechanism. *Asip1* overexpression affects adult melanophores in a position-dependent manner. Thus, *Asip1* overexpression induces a drastic reduction in number of differentiated



354 melanophore, but has no effect on those localized ventral to the horizontal myoseptum,  
 355 despite the fact that *Asip1* levels in ventral stripe regions 1V and 2V of transgenic fish were  
 356 as high as those recorded in the dorsal regions of transgenic fish. This dorso-ventral pattern,  
 357 although the second to be recognized in zebrafish, is likely to be an ancestral one, given the  
 358 ubiquitous nature of countershading in vertebrates. It seems that the zebrafish exhibits an  
 359 ancestral dorso-ventral pigment pattern on which the striped pattern is superimposed. The  
 360 graded expression of *Asip1* seems to induce an inversely correlated expression pattern of  
 361 *Mitfa*, showing minimal expression levels in the ventrum and maximal in the dorsal region. In  
 362 contrast, the pattern of *Dct* and *Tyrp1b* is more complex, being lowest in the belly, but  
 363 otherwise not tightly-correlated to the *Mitfa* pattern. When the *Asip1* expression gradient is  
 364 disrupted in transgenic zebrafish so as to generate dorsal *Asip1* levels comparable to those  
 365 observed in the belly of transgenic or WT zebrafish, the expression pattern of both  
 366 melanogenic enzymes and the transcription factor *Mitfa* is also disrupted. These observations  
 367 are consistent with our previous study, which showed that local injection of *Asip1* capped  
 368 mRNA on dorsal dark skin of turbot decreased the expression of *Tyrp1b* (Guillot et al., 2012).  
 369 Furthermore, the effects were only detectable during metamorphosis and then maintained  
 370 throughout adulthood, as transgenic zebrafish never developed the 2D dark stripe on the  
 371 dorsum that usually develops with growing size.

372 Our observations indicate that the pattern change derives from both an inhibition of  
 373 melanisation, directly comparable to the mechanism of counter-shading documented in  
 374 mammals, but importantly also from a novel mechanism regulating the number of  
 375 melanocytes, and thus the ratio of melanocytes and other pigment cells. Thus, we have shown  
 376 in zebrafish that *Asip1*'s role in regulation of melanocyte melanisation is conserved. In  
 377 rodents, ASIP function is generally stated to act via repression of melanocyte differentiation,  
 378 specifically expression of melanogenic enzymes like Dct and Tyrp1 (Millar et al 1995,  
 379 Candille et al, 2004, Manceau et al, 2011). Likewise, in rodent cell culture, ASIP inhibits  $\alpha$ -  
 380 MSH-induced eumelanisation of melanoblasts (Aberdam et al., 1998; La Pape et al., 2008;  
 381 Sviderskaya et al., 2001). Sviderskaya et al. (2001) showed that melanocortin agonists

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stimulate differentiation of immortal cell lines of murine melanoblasts but inhibited growth; however, when these cells were cultured with agouti, melanoblast differentiation was inhibited and growth stimulated. ~~Furthermore, it has been shown that proliferation and differentiation of mouse epidermal melanocytes are controlled by genetic factors (semi-dominant genes (ASIP) and coat color genes (TYROSINASE) as well as local tissue environment in response to hormones (e.g., MSH) and growth factors (e.g., bFGF) (Hirobe 1982, 1992; Tamate, et al., 1986).~~

The mechanism by which *Asip1* decreases *Dct* and *Tyrp1b* expression and inhibits melanophore differentiation is likely to be similar to that described in mammalian species. In mammals, ASIP antagonism of MC1R signaling induces the eumelanin-pheomelanin switch by lowering cAMP-response element binding protein (CREB)-dependent expression of *MITF* that stimulates transcription of *TYROSINASE*, *TYRP1* and *DCT* (Bertolotto et al., 1998a; Bertolotto et al., 1998b). In fish, MC1R signaling is also involved in the control of pigmentation since nonsense mutations in MC1R have been reported to result in albinism (Gross et al., 2011). Consistent with the antagonistic role in mammals, *Asip1* blocks  $\alpha$ -MSH-induced melanin dispersion in medaka scale melanophores by acting as a competitive antagonist at MC1R (Cerdá-Reverter et al., 2005). In the sea bass (*Dicentrarchus labrax*), MC1R is constitutively activated and the endogenous melanocortin antagonists work as inverse agonists thus decreasing the constitutive activity of the receptor (Sánchez et al., 2012). It is, therefore, plausible that the constitutive activation of MC1R leads to dorsal melanogenesis but ventral expression of *Asip1* blocks the constitutive activity thus inhibiting ventral melanogenesis. When *Asip1* is overexpressed in transgenic zebrafish, *Asip1*/MC1R interaction in the dorsal striped region results in reduced *Mitfa* expression that results in a lowering of the expression levels of melanogenic enzymes.

At least in the very dorsal most regions, our data suggests this reduction in melanisation is concomitant with a diminution of the number of melanocytes, so that the ventralization of the dorsal region is partially dependent upon a reduction in the ratio of different cell-types. This

might also be dependent upon the levels of *Mitfa* expression, since at low levels melanocyte specification from MSCs and/or survival or proliferation of melanoblasts might be affected (Lister et al., 1999; Carreira et al., 2006).

In contrast to a clear role in post-metamorphic (adult) melanocyte development and pattern formation, our data gives no evidence for a role for *Asip1* in embryonic melanocyte development nor in larval (pre-metamorphic) pigment pattern formation, since we saw no differences in the number of melanophores at 5 and 15 dpf, and since the embryonic WT expression pattern is totally uncorrelated with pigment pattern.

Our results lead us to propose also a differential regulation of adult melanophore differentiation, proliferation and/or survival depending on their position. The number of ventral melanophores (1V and 2V) was unaffected by *Asip1* overexpression. In addition, pigmentation of fin stripes of *Asip1* transgenic fish was indistinguishable from that observed in WT fish. The independence of some mechanisms underlying the control of body and fin pigment patterns has been also reported in some zebrafish mutants like *ssShady/Ltk* (Lopes et al., 2008). Similar to *Asip1* transgenic fish, *ssShady/Ltk* zebrafish exhibit a severe disruption of the body stripe pattern but keep intact the fin stripes. It seems likely that some subpopulations of melanophores or their latent precursors are not susceptible to the effects of *Asip1*; our expression data indicated that, although expressed in all fin tissue, *Asip1* expression levels did not show a graded expression pattern in any that we examined, and furthermore there was no correlation between *Asip1* expression levels and the location of the fin. Thus, fin pigment pattern formation appears to be largely independent of *Asip1*.

Cell culture studies exploring the actions of MIF have reported stimulation of iridophore proliferation, in addition to inhibition of melanocyte development (Bagnara and Fukuzawa, 1990; Zuasti et al., 1992). Suggestively, a common origin of both post-embryonic iridophore and melanophore has been recently proposed from so-called MSCs (Budi et al., 2011). The dorsalmost band of iridophores appears expanded in the dorsal area of *Asip1* transgenic

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zebrafish, strongly contributing to the ventralised appearance of their dorsal skin. However, our studies of sectioned adult fish indicate that these cells are present in WTs, but lie underneath, and are obscured by, the dorsal melanophores. This is consistent with the extensive distribution of these cells throughout stripe and interstripe regions as demonstrated by electron microscopy (Hirata et al., 2003; Hirata et al., 2005). We analyzed the distribution in adult skin of xanthophores and iridophores using the molecular markers *xXdh* (Parichy et al., 2000) and *Ltk* (Lopes et al., 2008) respectively. Although expression of both *xXdh* and *Ltk* genes was widespread throughout the skin, we saw no correlation of either to the expression levels of *Asip1*. Thus, our data does not support an agouti-induced switch of MSCs to iridophores. Taken together, we conclude that development of xanthophores and iridophores may be largely independent of *Asip1* levels, although since we were unable to count the number of xanthophores or iridophores in adult fish, we cannot rule out a more subtle effect on their patterning. We suggest that the more pronounced band of iridophores in the transgenics results at least largely from the underlying cells becoming more visible in the absence of stripe 2D melanocytes.

From an evolutionary point of view we note both the conserved and novel aspects of the mechanisms of *Asip1* function in establishing the dorso-ventral pigment pattern in these divergent vertebrates. In mammals, *ASIP* regulates—the ~~switch between eumelanin/pheomelanin synthesis as well as the proliferation and/or differentiation in of~~ their only chromatophore type, the melanocyte ~~(Hirobe 1982, 1992; Tamate, et al., 1986)~~. In contrast, in fish a similar dorso-ventral pigment patterning process comes about by regulating the ratio of different pigment cell-types in different dorso-ventral zones, as well as by regulating melanocyte differentiation. Nevertheless, a further role for ASIP in mice in regulating specification, proliferation or survival of melanoblasts has not been ruled out experimentally (Hirobe 1982, 1992; Tamate, et al., 1986). In the light of our data, we suggest that a rigorous examination of this issue of pigment cell numbers (melanocytes, but in fish

also iridophores) using transgenic markers in both mouse and zebrafish will be important to clarify the degree to which a conserved mechanism of ASIP-dependent control of melanoblast numbers may contribute to dorso-ventral pigment pattern differences throughout the vertebrates.

In the evolution of mammals, the loss of pale coloured chromatophores appears to have allowed selection for biochemical mechanisms to produce pale versions of melanin to replace the function of light reflecting chromatophores. Concomitantly, the function of ASIP/MC1R interaction evolved to control the spatial-temporal switch in the synthesis of melanins.

~~Therefore, it seems that the ancestral function of ASIP was to control dorso-ventral difference acting on the ratio of different pigment cell types in different dorso-ventral zones and on the pigment cell number and /or differentiation, before the phenomenon of type-switching evolved.~~

In conclusion, we demonstrate for the first time the existence of a graded expression of *Asip1* along the dorso-ventral axis in adult fish, with the highest levels in the belly. This gradient functions to establish a dorso-ventral pigmentation pattern, because the disruption of the dorso-ventral expression gradient in transgenic fish overexpressing *Asip1* leads to phenotypic changes including the elimination of dorsal melanin pigmentation. *Asip1* seems to affect both melanocyte numbers and their differentiation in adult fish, but has no effect on embryonic pigment pattern. Thus, ASIP's role in establishing dorso-ventral pigment pattern is not unique to mammalian species, and it is likely that its function is conserved among vertebrates. However, *Asip1* overexpression had only modest effects on the general striped pattern of the body demonstrating that zebrafish has two largely independent pigment patterning mechanisms, an ancestral dorso-ventral pigment patterning process, onto which the striping mechanism is superimposed. Our study opens new ways for investigating the factors that control regional *Asip1* expression and which may have a role in generating natural variations in pigment pattern formation in fish. Transgenic zebrafish overexpressing *Asip1* will be a key

487 tool for the study of adult pigmentation in fish.

488

## 489 MATERIALS AND METHODS

490

### 491 Zebrafish strains and husbandry

492 Zebrafish were cultured as previously described (Westerfield, 2007). Experiments were  
493 performed with a TU-WT strain (Tuebingen (TU), Nüsslein-Volhard Lab). Post-embryonic  
494 stages were estimated by both standardized length (SL) measurements according to Parichy et  
495 al. (2009) and time post fertilization in hours or days (hpf or dpf).

496

### 497 Transgenic zebrafish lines

498 Three independent transgenic zebrafish lines (*Tg(Xla.Eef1a1:Cau.Asip1)iim03*,  
499 *Tg(Xla.Eef1a1:Cau.Asip1)iim04* and *Tg(Xla.Eef1a1:Cau.Asip1)iim05*) were generated with  
500 the *Tol2* transposon system. The *Tol2* vector was kindly provided by Dr. Kochi Kawakami  
501 (2007). The goldfish *Asip1* gene sequence was published by Cerdá-Reverter and co-workers  
502 in 2005 (NCBI reference sequence: XM\_001334910.3). To obtain a full-length cDNA  
503 sequence of *Asip1* from goldfish skin we used the *Asip1* 5'-ATGCATCCGTCATTGTTG-3'  
504 and 5'-TCAGCATTTTCGGGTAAC-3' primers (Cerdá-Reverter et al., 2005). The *Asip1* PCR  
505 product was subcloned into pGEMT easy vector with BamH I and Not I restriction sites.  
506 PGEMT-*Asip1* goldfish was digested with BamH I and Not I and subcloned into  
507 *pT2AL200R1506* cut with the same restriction sites. The *Tol2*-transposon-based vector  
508 *pT2AL200R150G* contains the *eGFP* gene under the control of elongation factor 1-alpha  
509 (EF1-alpha), a constitutive promoter. We replaced the *eGFP* gene with *Asip1* gene. A total of  
510 250 pg of construct and synthetic 5' capped mRNA (150 pg) encoding a transposase were co-  
511 injected into WT embryos at the one- or two-cell stage, with 1% of phenol red as tracer.

512 The pigment pattern of all adult mosaic fish (F0) showed a pronounced phenotype, with paler  
513 head and body than WT fish (Fig. S5). Positively identified F1 siblings were mated. The F2  
514 embryos were grown to sexual maturity and individuals F2 were mated to wild-type fish. The  
515 F3 progeny were analyzed for the transgene. Identified homozygous F2 were mated to each  
516 other to produce a large stable homozygous (F3) population.

517

#### 518 **Ethics statement**

519 All zebrafish experiments were approved by the National Advisory Committee for Laboratory  
520 Animal Research Guidelines licensed by the Spanish Authority (1201/2005) and conformed  
521 to the European Convention for the Protection of Animals used for Experimental and  
522 Scientific Purposes (ETS N° 123, 01/01/91).

523

#### 524 **Zebrafish tissues**

525 Eggs and larvae from fish aged 0–24 hpf and 2–7 dpf and adult tissues from eye, dorsal and  
526 ventral skin, intestine, heart, liver, brain, gill, kidney, fat, gonads and muscle were sampled  
527 for RT-PCR. Two groups of eight fish were establish, a WT group and a group of transgenic  
528 fish that overexpress the *Asip1* gene, *Tg(Xla.Eef1a1:Cau.Asip1)iim05*, see below. For each  
529 group, dorsal, primary and secondary stripes (1D, 1V and 2D, 2V, respectively) and belly skin  
530 samples were excised with a scalpel for qRT-PCR (see Fig. 1I,J for location of skin sample  
531 points). Animals were anaesthetized with 0.02% tricaine, adults were rapidly decapitated and  
532 all samples were frozen at -80 °C until analysis. RNA was extracted for comparison of  
533 expression levels. Larvae from 30 hpf were anaesthetized and fixed in 4% paraformaldehyde  
534 for *in situ* hybridization. Individual larvae, metamorphic and adult stages (SL 3.6, 6.3, 13 and  
535 25 mm or 5, 15, 60 and 210 dpf, respectively) were obtained for melanophore counts.

536

#### 537 **RNA isolation, RT-PCR and qRT-PCR**

538 Total RNA was isolated from zebrafish tissues using Trizol reagent (Invitrogen) according to  
 539 manufacturer's instructions and concentration determined by absorbance at 260 nm using  
 540 NanoDrop 2000 (Thermo scientific). A total of 100 ng of messenger RNA (mRNA) was  
 541 reverse transcribed using a first-strand complementary DNA (cDNA) synthesis kit  
 542 (Fermentas).  
 543 The ontogenic and tissue expression pattern of *Asip1* mRNA was determined by RT-PCR.  
 544 Quantification of *Asip1*, *microphthalmia associated transcription factor (Mitfa)*, *dopachrome*  
 545 *tautomerase (Dct)*, *tyrosinase-related protein 1b (Tyrp1b)*, *leukocyte tyrosine kinase (Ltk)*,  
 546 and *xanthine dehydrogenase (Xdh)* mRNA expression was determined by quantitative real-  
 547 time PCR (qRT-PCR) in the skin of WT and *Tg(Xla.Eef1a1:Cau.Asip1)iim05*, *Asip1*  
 548 transgenic fish line and for temporal expression *Asip1* pattern in wt zebrafish . The sequence-  
 549 specific primer sets are listed in Table S1. *Asip1* primer sets for zebrafish were designed from  
 550 the mRNA sequence published in the Genbank database (NM\_001128801.1, (Klovins and  
 551 Schioth, 2005). *β-Actin (Actb)* was used as the internal reference to test the quality of the  
 552 cDNA. A negative control was incorporated by replacing the cDNA template with molecular  
 553 biology-grade water. The qRT-PCR reaction was set up in triplicate, containing 2 µl of first-  
 554 strand cDNA, 10 pmol of each forward and reverse primer and SYBR PCR mix (Fermentas)  
 555 to a final volume of 20 µl. qRT-PCR was performed with an AB 7300 Real-Time PCR  
 556 System. The thermal cycle protocol for SYBR Green-based qRT-PCR for all primer sets was:  
 557 denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.  
 558 Each set of skin samples from each group was tested five or seven times. The housekeeping  
 559 gene *elongation factor-1 alpha (Ef-1 alpha)* was used as internal reference to normalize the  
 560 cDNA template between tissue and development stage samples. Normalized relative  
 561 quantities of mRNA expression were calculated with the mathematical method of  $\Delta\text{Ct}$  (Livak  
 562 and Schmittgen, 2001). The melting curves of the products were verified to confirm the  
 563 specificity of PCR products.



564

565 ***In situ hybridization***

566 For whole-mount *in situ* hybridization, samples at 1, 3, and 6 dpf were fixed in 4%  
567 paraformaldehyde (PFA) containing 1% DMSO in 1 × PBS overnight at 4°C and stored in  
568 100% methanol at -20°C. Whole-mount *in situ* hybridization was performed using  
569 digoxigenin-labeled antisense probes as previously described (Rotllant et al., 2008). Antisense  
570 and sense riboprobes were made from linearized full-length zebrafish and/or goldfish *Asip1*  
571 cDNA. For plastic sections, embryos with ISH staining were fixed, dehydrated and epon-  
572 embedded, and cut into 4 µm ventral longitudinal sections and mounted with EUKIT as  
573 previously described (Westerfield et al., 2007) and imaged.

574 *In situ* hybridization of adult (210dpf) zebrafish followed (Cerdá-Reverter et al., 2000).  
575 Samples were fixed in 4% paraformaldehyde, 0.1 M phosphate buffer, for 2 days at 4°C,  
576 dehydrated, and embedded in Paraplast (Sherwood, St Louis, MO). Serial 10 µm rostro-  
577 caudal transverse sections were cut using a rotary microtome. Sections were mounted on 3-  
578 aminopropyltriethoxysilane (TESPA)-treated slides, air-dried at room temperature (RT)  
579 overnight and stored at 4°C under dry conditions and used for hybridization within one  
580 month. Adjacent sections were stained with hematoxylin–eosin (HE) to identify physiological  
581 structures.

582

583 **Melanophore counts**

584 To test whether the melanophore pattern of transgenic fish is different from that of control  
585 fish, we quantified melanised melanophores in both groups. The region selected for counting  
586 melanophores depended on the stage of development. In early and metamorphic stages (5 and  
587 15 dpf, respectively), we counted total melanophores from head to tail in a dorsal view. For  
588 the metamorphic stages, the melanophores visible in the horizontal myoseptum were also

included. For adult fish (60 and 210 dpf), melanophores within a 1 mm<sup>2</sup> area were counted in multiple defined positions: from a dorsal view, on the head (head areas) and on the dorsal areas (edge of the head to the dorsal fin); from a lateral view, on the 2D, 1D, 1V and 2V areas (anterior areas (pectoral to pelvic fin) and posterior areas (end anal fin), ; and from a ventral view on belly area (pectoral to pelvic fin) (see Fig. 2 A,B). To analyze the number of melanophores, fish were immersed in fish water solution containing 10 mg/ml epinephrine (Sigma) for approximately 30 min to contract melanosomes. Afterwards, fish were anesthetized and imaged. Melanophores were counted manually utilizing Adobe Photoshop CS2 software. The number of melanophores was plotted against the area in mm<sup>2</sup>

## Statistics

Data are expressed as mean  $\pm$  standard error of the mean (SEM). Comparisons between numerical data were evaluated by one- or two-way ANOVA with the Tukey or Dunnett post hoc test. A *p* value less than 0.05 (asterisks and letters) was considered statistically significant. Statistics data are similar if share at least one letter. Statistical analyses and figures were performed with GraphPad Prism 5.0.

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## FIGURE LEGENDS

**Figure 1.** The *Asip1* gene is dynamically expressed during zebrafish development. (A) Normalized gene expression levels of zebrafish *Asip1* throughout embryonic, larval and adult zebrafish development. (B) Analysis of differential dorsal-ventral *Asip1* gene expression in 15, 30 and 60 dpf WT zebrafish. *Asip1* starts to be differentially expressed in ventral non-pigmented skin or dorsal pigmented skin at 30 dpf WT zebrafish.

852 The relative expression of total *Asip1* mRNA was determined by real-time qRT-PCR. Shown  
853 are log 10 transformed  $\Delta$ Ct values of *Asip1* relative to *Ef-1 alpha*. Data are the mean  $\pm$  SEM  
854 from eight samples after triplicate qRT-PCR analysis. Data are the mean  $\pm$  SEM from eight  
855 samples after triplicate qRT-PCR analysis. Superscripts a, b and c indicate statistical  
856 differences ( $P < 0.05$ ) in gene expression levels among developmental stages (A, B).  
857 Superscripts \* indicate statistical differences ( $P < 0.05$ ) in gene expression among skin region  
858 (B) at the same developmental stage (statistics data are similar if share at least one letter).

859

860 **Figure 2.** Spatial expression of *Asip1* transcripts in larvae and adult zebrafish. (A) lateral and  
861 (B) ventral view of whole-mount *in situ* hybridization of *Asip1* expression of 3 dpf wild-type  
862 zebrafish larvae. Staining with the *Asip1* antisense probe shows expression in bilaterally  
863 symmetric regions posterior to the eye (arrow heads). (C-D) Ventral longitudinal sections (4  
864  $\mu$ m thick) of epon-embedded, whole mount *Asip1* *in situ* hybridisations in 3dpf (C) and 6dpf  
865 (D) zebrafish identify *Asip1* expression as being in the developing opercle. Anterior to the  
866 left. (E-H) Rostro-caudal transverse sections (10  $\mu$ m thick) of paraffin-embedded, *in situ*  
867 hybridization of *Asip1* expression in 210 dpf (360 mm total body length) zebrafish. The  
868 section in E has been stained with hematoxylin-eosin and shows the typical location of  
869 dermal melanophores (brown arrows) and iridophores (blue arrows) in the zebrafish dermis.  
870 G and H are sequential sections of the fish shown in E. *Asip1* transcripts are expressed in the  
871 dermal iridophores located in the most ventral interstripe (interstripe X1V) (G) and stripe  
872 (stripe 2V) (H). The section in (F) shows the expression of *Asip1* transcripts in the  
873 intraperitoneal membrane region where a reflecting iridophore layer is located. Abbreviations:  
874 ch, ceratohyal; e, eye; ep, epidermis; m, muscle; op, opercle; p, pharyngeal arches; pc,  
875 peritoneal cavity; pm, peritoneal membrane; pf, pectoral fin; Scale bars: (A,B) 0,25 mm; (C)  
876 25  $\mu$ m; (D) 50  $\mu$ m; (E,H) 20  $\mu$ m; (F) 80  $\mu$ m; (G) 60  $\mu$ m.

877

878 **Figure. 3** Adult pigment pattern of wild-type (WT) and transgenic agouti fish (*Asip1*-Tg; line  
879 *Tg(Xla.Eef1a1:Cau.Asip1)iim05*). Lateral (A, B), dorsal (C, D), anterior-lateral (E,F,J,I) and  
880 dorsal head close-up views (G, H) of 210 dpf zebrafish. (A, B) WT fish have a pattern with  
881 dark stripes and light interstripes. Letters indicate the name of each dark stripe rich in mature  
882 melanophores: the two primary stripes are designated 1D and 1V, and the two secondary  
883 stripes 2D and 2V. *Asip1* overexpression resulted in a lighter pigment pattern. In *Asip1*-Tg  
884 fish, the 2D stripe is absent, and there are fewer melanophores in the 1D stripe. In areas  
885 lacking melanophores, xanthophores become more evident, and the fish looks much paler and  
886 yellower (B, D). *Asip1*-Tg fish show a dramatic reduction in melanophore number over the  
887 flank (E, F). In WT, melanophores are distributed at the end of scales and over the head; in  
888 *Asip1*-Tg, the head is clearly hypopigmented, xanthophores and iridophores are more evident  
889 and the scales largely lack melanophores (G, H). (I,J) location of skin sample points (white  
890 boxes) of 210 dpf adult WT (I) and *Asip1*-Tg (J) fish. (K,L) normalized gene expression  
891 levels of zebrafish *Asip1* in skin samples from dorsum, primary stripes (1D and 1V),  
892 secondary stripes, (2D and 2V) and belly of 210 dpf adult WT (I) and *Asip1*-Tg (J) fish.  
893 Shown are log 10 transformed  $\Delta\text{Ct}$  values of *Asip1* relative to *Ef1-alpha*. Data are the mean  $\pm$   
894 SEM from eight samples after triplicate qRT-PCR analysis. Superscripts a, b and c indicate  
895 statistical differences ( $P < 0.05$ ) in gene expression levels among skin region (statistics data are  
896 similar if share at least one letter). Scale bar: 0.5 cm.

897  
898 **Figure. 4** Distribution (A-B) and number (C,D) of melanophores in WT and *Asip1*-Tg in  
899 60dpf (C) and 210dpf zebrafish (A, B, D). Lateral views of 210 dpf WT (A) and *Asip1*-Tg (B)  
900 showing the fish body regions selected for melanophore count are displayed (A, B). At 60dpf  
901 (C) black melanophores are organized in a longitudinal stripe pigment pattern scattered over  
902 the flank, head and ventral areas. At this stage, wild-type fish show the first two primary dark  
903 stripes, 1D and 1V (C, inset); stage-matched *Asip1*-Tg fish showed significantly lower

904 numbers of melanophores in the head, dorsal area and 1D stripe region but not in ventral  
 905 areas (1V stripe and belly) than their WT siblings ( $p<0.001$ ). At 210 dpf (D), additional  
 906 melanophore stripes are formed (D, inset). *Asip1*-Tg fish showed significantly reduced  
 907 numbers of melanophores in the head, dorsal area, 1D and 2D stripe region but not in ventral  
 908 areas (1V and 2V stripe and belly) than WT siblings ( $p<0.001$ ). Melanophores within a 1 mm<sup>2</sup>  
 909 area were counted in the in the dorsal head (head), dorsal lateral back (dorsal), anterior and  
 910 posterior 1D and 1V stripes and in the lateral belly of 60dpf WT and *Asip1*-Tg fish and in the  
 911 dorsal head (head), dorsal lateral back (dorsal), anterior 2D and 2V stripes, anterior and  
 912 posterior 1D and 1V stripes and in the lateral belly of 210dpf WT and *Asip1*-Tg fish. Data are  
 913 the mean  $\pm$  SEM, n=8. Asterisks indicate significant differences ( $P<0.05$ ) between WT and  
 914 *Asip1*-Tg fish. Scale Bars: 1 mm.

915  
 916 **Figure. 5** qRT-PCR analysis of pigment cell markers in the skin of WT and *Asip1*-Tg  
 917 zebrafish. mRNA expression levels of *microphthalmia-associated transcription factor a*  
 918 (*Mitfa*)(A), *dopachrome tautomerase* (*Dct*)(B), *tyrosinase-related protein 1* (*Tryp1b*)(C),  
 919 *xanthine dehydrogenase* (*Xdh*)(D) and *leukocyte tyrosinase kinase* (*Ltk*)(E) were determined  
 920 in skin samples from dorsum, primary stripes (1D and 1V), secondary stripes, (2D and 2V)  
 921 and belly of 210dpf adult fish. Shown are log 10 transformed  $\Delta$ Ct values of *Mitfa*, *Dct*,  
 922 *Tryp1b*, *Xdh* and *Ltk* relative to *Efl-alpha*. Data are the mean  $\pm$  SEM from eight samples  
 923 after triplicate qRT-PCR analysis. Asterisk indicate significant differences ( $P<0.05$ ) between  
 924 WT and *Asip1*-Tg fish. Superscripts a, b and c indicate statistical differences ( $P<0.05$ ) in gene  
 925 expression levels among skin region (statistics data are similar if share at least one letter).  
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